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Quantitative detection of HER2 protein concentration in breast cancer tissue does not increase the number of patients eligible for adjuvant HER2-targeted therapy

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Abstract. Human epidermal growth factor receptor-2 (HER2) is overexpressed in 15-20% of breast cancer patients and is associated with an aggressive tumor and a poor prognosis. Currently, patients are selected for adjuvant HER2-targeted therapy based on HER2 status by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). In this study, we assessed the clinical significance of tissue HER2 status determined by a quantitative immunoassay using ADVIA Centaur. We investigated the hypothesis that the clinical outcome is worse in a group of patients defined as tissue HER2-positive only by Centaur, but not treated with adjuvant HER2-targeted therapy, compared to patients defined as HER2-positive by IHC/FISH and therefore treated with adjuvant HER2-targeted therapy. Tumor tissue was obtained at primary surgery from 415 breast cancer patients between 2004 and 2010. HER2 status was determined by quantitative immunoassay of fresh-frozen tissue and by IHC/FISH of corresponding paraffin-embedded tissue. We compared the clinical outcome in four groups of patients defined by tissue HER2 status and adjuvant HER2-targeted therapy. The final analysis included 379 patients after a median follow-up of 3.9 years for invasive disease-free survival (IDFS) and 4.2 years for overall survival (OS). The quantitative Centaur assay defined a greater number of patients (100 patients, 26.4%) as HER2-positive than IHC/FISH (63 patients, 16.6%) (P<0.0001). No significant difference in IDFS (P=0.159) and OS (P=0.150) was observed among the four groups of patients. However, in the IHC/FISH-positive group without adjuvant HER2-targeted therapy (group 2), a significantly greater number of events was found compared to the Centaur-positive group without adjuvant HER2-targeted therapy (group 3) for both IDFS (P=0.025) and OS (P=0.020). Quantitative HER2 determination by Centaur did not define a new group of patients eligible for HER2-targeted therapy. Currently, tissue HER2 status defined by IHC/FISH analysis remains the gold standard.

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Introduction

Breast cancer is the leading cancer among women in the industrialized world and 15-20% of breast cancer tumors feature an overexpression and/or amplification of human epidermal growth factor receptor-2 (HER2). HER2, also termed HER2/neu, ErbB2 or p185HER2, is one of four tyrosine kinase receptors of the HER family which includes HER1 (EGFR), HER2, HER3 and HER4. The HER2 gene is located on chromosome 17 and encodes HER2, which is a 185-kDa glycoprotein composed of an intracellular tyrosine kinase domain, a transmembrane domain, and an extracellular domain with a yet unknown ligand (1). Activation of the HER2 pathway is presumably driven by the binding of heregulins to HER3 and HER4 or EGF to HER1 and the subsequent hetero-dimerization with HER2, which leads to the activation of the downstream pathway (2).

Overexpression of the HER2 protein and/or amplification of the HER2 gene leads to tumor cell proliferation and is associated with an aggressive tumor and a poor prognosis (3,4). Furthermore, HER2 overexpression/amplification predicts the effect of HER2-targeted therapy (e.g., trastuzumab and lapatinib) in combination with chemotherapy in both the metastatic and adjuvant setting, and several studies have demonstrated that the addition of trastuzumab reduces the risk of recurrence in HER2-positive breast cancer patients by approximately 50% (5-7).

Several studies have reported the discordance of HER2 status between primary and recurrent disease years after the primary treatment in 3-14% of the cases (8-10). This has led to the hypothesis that an additional group of patients may benefit from HER2-targeted therapy in the adjuvant setting. However, it remains unclear whether the observed discordance of HER2 status is due to heterogeneity of the primary tumor, acquired HER2 expression during the course of the disease, or limited sensitivity of the assay leading to misclassification of a modest,

but clinically relevant HER2 overexpression. Currently, patients are selected for adjuvant HER2-targeted therapy based on immunohistochemistry (IHC) and fluorescence- or chromogenic *in situ* hybridization (FISH, CISH) (11). In addition to these semi-quantitative tests, quantitative real-time PCR and microarray-based RNA expression analysis of HER2 have emerged over the past decade, delivering quantitative estimates of HER2 DNA and RNA expression (12-14).

In this study, tissue HER2 status was determined by a quantitative immunoassay using ADVIA Centaur. This assay is able to analyze a larger tumor amount, whereby the influence of tumor heterogeneity is reduced compared to IHC/FISH. By using this method, an additional 9% of patients were classified as HER2-positive compared to the conventional IHC/FISH methods as reported in a previous study by Olsen *et al* (15). The clinical relevance of this information however, is unknown and therefore, the aim of the present study was to perform a clinical evaluation of the quantitative Centaur assay. We wished to examine the hypothesis that the clinical outcome is poorer in a group of patients defined as tissue HER2-positive by Centaur only, but not treated with adjuvant HER2-targeted therapy, compared to patients defined as HER2-positive by IHC/FISH and therefore treated with adjuvant HER2-targeted therapy.

Patients and methods

Study population and patient samples. This prospective cohort study was performed in a single center cancer hospital. Women eligible for primary surgery for breast cancer stages I-IIIA were included after written informed consent. The study was approved by the Regional Scientific Ethics Committee for Southern Denmark (project identification number S-VF-20040101). Tumor tissue samples and autologous reference tissue samples were obtained from 415 breast cancer patients between 2004 and 2010. Surgery was performed in accordance with the guidelines from the Danish Breast Cancer Cooperative Group (DBCG).

The tissue samples were obtained within 1 h after surgery. One part of the sample was fixed in formalin and paraffin-embedded for IHC/FISH analysis. The other part of the sample was snap-frozen in liquid nitrogen and stored in a local biobank at -80˚C for later Centaur analysis. A dedicated pathologist verified the presence of tumor tissue in the tumor sample and the lack of tumor tissue in the autologous reference tissue. Reference tissue was taken at least 1 cm away from tumor tissue whenever possible.

A total of 36 patients were excluded from the final analysis due to advanced disease, benign pathology, neoadjuvant chemotherapy, or missing tumor tissue (flow diagram, Fig. 1). The mean age of the remaining 379 patients was 60 years (34-91 years). A total of 272 patients (71.8%) were treated with breast-conserving surgery, while 107 (28.2%) had a mastectomy. Only 17 patients (4.5%) had bilateral synchronous breast cancer. The majority of patients (330 patients, 87.1%) had invasive ductal carcinoma, 25 patients (6.6%) had invasive lobular carcinoma, and 24 patients (6.3%) had other types of breast cancer.

End points. End points were defined according to the standard definitions by Hudis *et al* (16) Invasive disease-free survival (IDFS) was defined as the time from primary surgery to one of the following events: ipsilateral invasive breast tumor recurrence, regional invasive breast cancer recurrence, distant recurrence, death by any cause, contralateral invasive breast cancer, or second primary non-breast invasive cancer. This definition excludes all types of carcinoma *in situ* [ductal carcinoma *in situ (*DCIS), lobular carcinoma *in situ* (LCIS) and all *in situ* cancers of non-breast sites] and squamous or basal cell skin cancers. Overall survival (OS) was defined as the time from primary surgery to death by any cause (includes death from breast cancer, non-breast cancer or unknown causes).

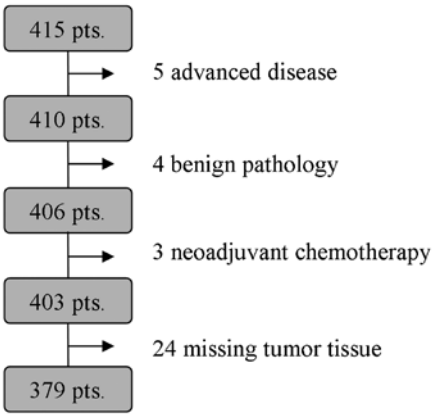

Figure 1. Diagram outlining the exclusion of patients. pts, patients.

Clinical and histological data. Pathological data were obtained from the DBCG database and verified in the local database at the Department of Pathology, Vejle Hospital, Vejle, Denmark. Clinical IDFS data were obtained from the local electronic health records and the nationwide online electronic health records holding data from all Danish hospitals. OS data were obtained from the nationwide Danish Civil Registration System, which contains basic personal data on all residents in Denmark.

Tissue HER2 determination. Homogenization of the tissue samples and determination of the tissue HER2 status and estrogen receptor (ER) status have been described in detail in a previous study of ours [Olsen *et al* (17)]. The quantitative detection of HER2 tissue concentration was determined using fresh-frozen tumor tissue and autologous reference tissue by means of commercially available HER2/neu assay using the ADVIA Centaur system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). This automated immunoassay uses two monoclonal antibodies (TA-1 and NB-3) for the detection of HER2 protein. The chemiluminescence signal is directly proportional to the quantity of HER2 protein in the sample. Each assay was controlled by two commercial controls (Siemens Healthcare Diagnostics) and one in-house serum pool. The assay shows an acceptable inter-assay coefficient of variation (CV) between 4.4 and 5.6%.

Tissue HER2 status was determined on paraffin-embedded tumor tissue using the IHC and FISH methods. The tumors were considered HER2-positive if defined IHC3$^+$ or IHC2$^+$ combined with FISH ≥2. IHC analysis was assessed by HercepTest™ (DakoCytomation, Glostrup, Denmark).

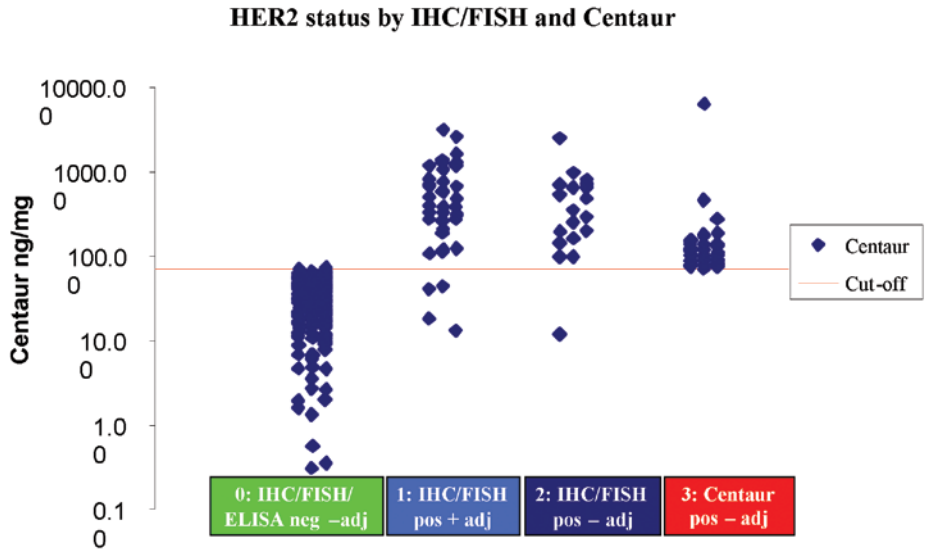

Figure 2. Distribution of ADVIA Centaur values according to the four different groups of patients defined by tissue HER2 status (IHC3+ or IHC2+ with FISH ≥2 or Centaur >72 ng/mg cut-off value), +/- adjuvant HER2-targeted therapy. neg, negative; pos, positive; adj, adjuvant HER2 targeted therapy.

IHC0 and IHC1+ were considered HER2-negative, whereas IHC3+ was defined as HER2-positive. IHC2+ was considered borderline and in these cases HER2 expression was further determined using the HER2 FISH pharmDx™ kit (DakoCytomation). The threshold for overexpression was a ratio equal to or exceeding 2.0 between the HER2 gene copy number and the chromosome 17 centromere.

ER staining was carried out using an anti-human ER monoclonal antibody (clone 1D5; DakoCytomation) and visualized by the SuperSensitive polymer-HRP IHC kit (Biogenex Laboratories Inc., San Ramon, CA, USA). Tumors with a nuclei staining ≥10% were considered ER-positive according to the contemporary DBCG guidelines.

Statistical methods. We enrolled 400 patients to detect a 25% absolute reduction in the risk of IDFS events from 45 to 20% with 80% power and a two-sided significance level of 5%. The statistical analyses were carried out using Stata version 11 software (StataCorp LP, TX, USA). Kaplan-Meier curves and the log-rank test were used to compare all time-to-event end points. Multivariate IDFS and OS analyses were performed using the Cox proportional hazards regression model. Fisher's exact test and McNemar's test were used to compare categorical data. ROC curves were used to investigate the cut-off for the quantitative Centaur assay. For all tests, two-sided P-values <0.05 were considered statistically significant.

Results

Patient characteristics. Final analysis included 379 patients. At the clinical cut-off date (September 19, 2011) the median follow-up was 3.9 years for IDFS and 4.2 years for OS. We compared the clinical outcome in four groups of patients defined by HER2 status (determined by IHC/FISH and Centaur) and adjuvant HER2-targeted therapy. The four groups were defined as follows: Group 0, patients defined as tissue HER2-negative by IHC/FISH and ADVIA Centaur and therefore not offered HER2-targeted therapy. Group 1, patients defined as tissue HER-positive by IHC/FISH and therefore offered HER2-targeted therapy. Group 2, patients defined as tissue HER2-positive by IHC/FISH, but not offered HER2-targeted therapy, as they were older than 60 years and therefore only received endocrine treatment when adjuvant treatment was required according to the recommendations by the contemporary DBCG guidelines. Group 3, patients defined as tissue HER2-positive by ADVIA Centaur, but not by IHC/FISH and therefore not offered HER2-targeted therapy.

Table I shows patient demographics and clinical characteristics in the four groups. Clinical prognostic factors were significantly better in group 3 compared to group 1 as regards tumor grade, axillary nodal status and ER status (P=0.001, 0.026 and <0.001, respectively; Fisher's exact test). Furthermore, Table I shows that the majority of patients in groups 1 and 2 were also defined as tissue HER2-positive by ADVIA Centaur. Likewise, differences in adjuvant treatment were observed as indicated in Table II. Significantly fewer patients in group 3 compared to group 1 received adjuvant chemotherapy (P<0.001; Fisher's exact test).

HER2 status determined by IHC/FISH and ADVIA Centaur. Paraffin-embedded and fresh-frozen tumor tissue samples were available from all 379 patients for IHC/FISH and ADVIA Centaur analyses. The cut-off value of 72 ng/mg protein for ADVIA Centaur HER2 positivity was determined on available autologous reference tissue from 371 out of 403 patients applying a 97.5% confidence interval (CI). As shown in our previous study [Olsen *et al* (17)], we found the median value of HER2 to be significantly higher in the tumor tissue (median, 42.3 ng/mg; range, 0-6158.2 ng/mg) than in autologous reference tissue (2.6 ng/mg; range, 0-862.8 ng/mg; P<0.0001; Wilcoxon signed rank test on 367 patients with both tumor and autologous reference tissue).

Using the cut-off value of 72 ng/mg, the quantitative ADVIA Centaur defined 100 out of the 379 patients (26.4%) as tissue HER2-positive, whereas only 63 patients (16.6%) were defined HER2-positive by the use of IHC/FISH (P<0.0001;

Table I. Demographics and clinical characteristics in the four groups of patients defined by HER2 status (determined by IHC/FISH and Centaur), +/- adjuvant HER2-targeted therapy.

	Group 0 HER2-neg - adj (n=274)		Group 1 HER2-pos IHC/FISH + adj (n=42)		Group 2 HER2-pos IHC/FISH - adj (n=21)		Group 3 HER2-pos Centaur - adj (n=42)		
Characteristic	No.	%	No.	%	No.	%	No.	%	P-value[a]
Age									
<40 years	6	2.2	4	9.5					
40-59 years	111	40.5	25	59.5	4	19.0	23	54.8	
≥60 years	157	57.3	13	31.0	17	81.0	19	45.2	0.091
Type of surgery									
Breast-conserving	192	70.1	30	71.4	16	76.2	34	81.0	
Mastectomy	82	29.9	12	28.6	5	23.8	8	19.0	0.443
Tumor type									
Ductal	236	86.1	38	90.5	19	90.5	37	88.1	
Lobular	21	7.7	2	4.8			2	4.8	
Others	17	6.2	2	4.8	2	9.5	3	7.1	1.000
Tumor grade									
Grade 1	68	24.8	1	2.4	1	4.8	12	28.6	
Grade 2	132	48.2	20	47.6	7	33.3	21	50.0	
Grade 3	56	20.4	17	40.5	11	52.4	6	14.3	
Unknown	18	6.6	4	9.5	2	9.5	3	7.1	**0.001**
Tumor size									
T1 ≤20 mm	130	47.4	17	40.5	9	42.9	24	57.1	
T2 >0 ≤50 mm	138	50.4	24	57.1	12	57.1	18	42.9	
T3 >50 mm	6	2.2	1	2.4					0.190
Nodal status									
N0, 0 nodes	127	46.4	18	42.9	11	52.4	26	61.9	
N1, 1-3 nodes	106	38.7	12	28.6	8	38.1	14	33.3	
N2, 4-9 nodes	28	10.2	8	19.0	2	9.5	1	2.4	
N3, ≥10 nodes	13	4.7	4	9.5			1	2.4	**0.026**
ER status									
Negative	38	13.9	18	42.9	6	28.6	3	7.1	
Positive	236	86.1	24	57.1	15	71.4	39	92.9	**<0.001**
HER2 IHC/FISH									
Negative	274						42		
Positive			42		21				NA
HER2 Centaur									
Negative <72 ng/mg	274		4		1				
Positive ≥72 ng/mg			38		20		42		NA

[a]Clinical prognostic factors in group 1 and group 3 were compared by Fisher's exact test. neg, negative; pos, positive; adj, adjuvant HER2 targeted therapy.

McNemar's test). The ADVIA centaur misclassified only 5 out of the 63 patients defined as tissue HER2-positive by IHC/FISH as shown in Table I. Fig. 2 shows the distribution of ADVIA Centaur values according to the four different groups; it should be noted that only five values in groups 1 and 2 are below the cut-off value of 72 ng/mg.

Table II. Adjuvant therapy in the four groups of patients defined by HER2 status (determined by IHC/FISH and Centaur), +/- adjuvant HER2-targeted therapy.

Adjuvant therapy	Group 0 HER2-neg - adj (n=274)		Group 1 HER2-pos IHC/FISH + adj (n=42)		Group 2 HER2-pos IHC/FISH - adj (n=21)		Group 3 HER2-pos Centaur - adj (n=42)		P-value[a]
	No.	%	No.	%	No.	%	No.	%	
Chemotherapy									
No	171	62.4	1	2.4	19	90.5	23	54.8	
CEF	42	15.3	13	31.0			4	9.5	
CMF	1	0.4	1	2.4	2	9.5			
EC + D	47	17.2	27	64.3			14	33.3	
DC	13	4.7					1	2.4	**<0.001**
HER2-targeted									
No	274	100.0			21	100.0	42	100.0	
Herceptin			28	66.7					
Neratinib[b]			4	9.5					
Lapatinib[b]			10	23.8					**<0.001**
Endocrine									
No	70	25.5	17	40.5	7	33.3	11	26.2	
Tam	45	16.4	7	16.7	1	4.8	6	14.3	
Tam + AI[c]	107	39.1	7	16.7	7	33.3	16	38.1	
AI	52	19.0	11	26.2	6	28.6	9	21.4	0.165
Radiotherapy									
No	49	17.9	4	9.5	6	28.6	6	14.3	
Yes	225	82.1	38	90.5	15	71.4	36	85.7	0.738

[a]Differences in the adjuvant therapy in groups 1 and 3 were compared by Fisher's exact test. [b]In combination with Herceptin. [c]Tamoxifen followed by an aromatase inhibitor. neg, negative; pos, positive; adj, adjuvant HER2 targeted therapy.

Table III. Univariate analysis of prognostic factors for IDFS and OS in the 379 patients.

Factor	IDFS P-value	OS P-value
Group	0.159	0.150
Age, <60 vs. ≥60 years	0.207	0.055
Tumor grade, grade <3 vs. grade 3	<0.001	<0.001
Tumor size, ≤ 20 vs. >20 mm	0.058	0.009
Lymph nodes, neg/pos	0.206	0.012
ER status, neg/pos	0.111	0.049
HER2 IHC/FISH, neg/pos	0.228	0.522
HER2 Centaur, <72 vs. ≥72 ng/mg	0.651	0.670

Univariate analysis was performed using the log-rank test. Median follow-up was 3.9 years for IDFS and 4.2 years for OS. neg, negative; pos, positive.

Invasive disease-free survival. Follow-up was available for all 379 patients in the final analysis. At a median follow-up of 3.9 years (0.3-6.9 years), there were 74 events in the four groups, 45 distant recurrence, 11 regional invasive breast cancer recurrence or ipsilateral/contralateral invasive breast cancer, 10 died from non-breast cancer/unknown cause, and eight had second primary non-breast invasive cancer. No significant difference in IDFS was found among the four groups (P=0.159; log-rank). Surprisingly, we found a significantly greater number of events in group 2 compared to group 3 (P=0.025; log-rank), with eight events in 21 patients in the IHC/FISH-positive group not receiving adjuvant HER2-targeted treatment (group 2) and only five events in 42 patients in the Centaur-positive group not receiving adjuvant HER2-targeted treatment (group 3) (Fig. 3).

Overall survival. At a median follow-up of 4.2 years (0.3-6.9 years), the four groups had a total of 39 events (29 died from breast cancer and 10 died from non-breast cancer/unknown cause). As for IDFS, no significant difference in survival was observed among the four groups (P=0.150; log-rank); however, a significantly higher number of deaths was observed in group 2 compared to group 3 (P=0.020; log-rank) (Fig. 4).

Univariate and multivariate analysis. Table III shows the results of the univariate analysis with dichotomized variables,

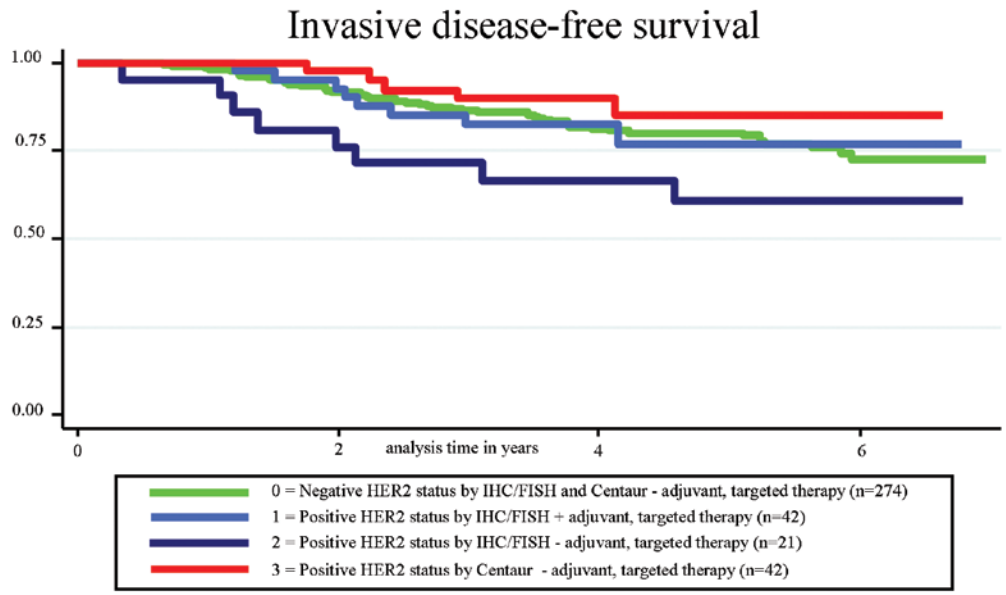

Figure 3. Kaplan-Meier plot showing invasive disease-free survival in the four groups of patients based on HER2 status (determined by IHC/FISH and Centaur), +/- adjuvant HER2-targeted therapy.

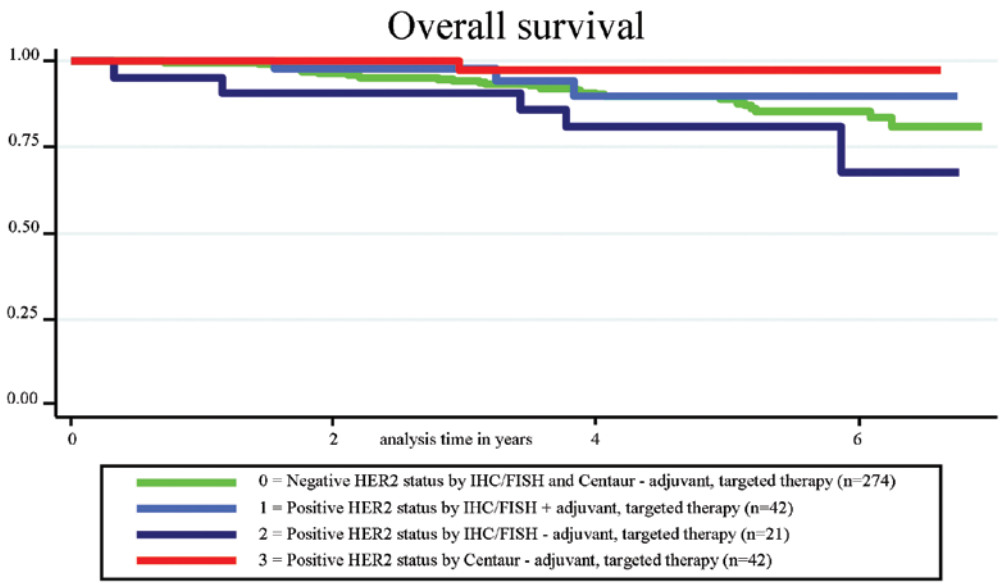

Figure 4. Kaplan-Meier plot showing overall survival in the four groups of patients based on HER2 status (determined by IHC/FISH and Centaur), +/- adjuvant HER2-targeted therapy.

identifying tumor grade (grade of <3 vs. grade 3), tumor size (≤20 vs. >20 mm), axillary node status (negative vs. positive) and estrogen receptor status (negative vs. positive) as statistically significant for OS, whereas only tumor grade was statistically significant for IDFS. HER2 status (negative vs. positive) was not significant in the univariate analysis, neither when determined by IHC/FISH nor by Centaur (<72 vs. ≥72 ng/mg).

The variables in the multivariate analysis included group (also representing HER2 status), age, tumor grade, tumor size, axillary node status and ER-status as outlined in Table IV. In the multivariate analysis for IDFS, the only independent prognostic marker was tumor grade (P=0.011). In the multivariate analysis for OS age (P=0.048), tumor grade (P=0.010) and axillary node status (P=0.025) were independent prognostic markers. Groups determined by HER2 status were not an independent prognostic factor in the above analysis.

ROC curve analysis. We also investigated the ability of the quantitative Centaur assay to discriminate between patients with or without recurrence. The ROC curve analyses resulted in an almost straight line with an area under the ROC curve of 0.49, which implies that the Centaur assay was not able to discriminate between patients at all. In accordance with this finding, there were no statistically significant differences observed between the mean Centaur value in patients with or without recurrence in any of the four groups of patients in this study (data not shown).

Table IV. Multivariate analysis of prognostic factors for IDFS and OS in 379 patients.

	IDFS		OS	
Factor	HR (95% CI)	P-value	HR (95% CI)	P-value
Group				
0, HER2-neg - adj	1.00		1.00	
1, IHC/FISH pos + adj.	0.94 (0.43-2.05)	0.879	0.72 (0.21-2.49)	0.602
2, IHC/FISH pos - adj.	1.49 (0.69-3.21)	0.306	1.29 (0.49-3.39)	0.602
3, Centaur pos - adj.	0.65 (0.26-1.62)	0.352	0.27 (0.04-2.01)	0.202
Age				
<60 years	1.00		1.00	
≥60 years	1.35 (0.83-2.22)	0.231	2.04 (1.00-4.15)	0.048
Tumor grade				
Grade 1, 2, unknown	1.00		1.00	
Grade 3	1.94 (1.16-3.24)	0.011	2.48 (1.24-4.97)	0.010
Tumor size				
≤20 mm	1.00		1.00	
>20 mm	1.32 (0.80-2.17)	0.624	1.80 (0.85-3.83)	0.124
Axillary node status				
Negative	1.00		1.00	
Positive	1.33 (0.82-2.17)	0.245	2.32 (1.11-4.83)	0.025
ER status				
Negative	1.00		1.00	
Positive	0.78 (0.43-1.41)	0.411	0.60 (0.28-1.29)	0.195

Statistical analysis was performed by the Cox regression analysis. Median follow-up was 3.9 years for IDFS and 4.2 years for OS. neg, negative; pos, positive; adj, adjuvant HER2 targeted therapy.

Discussion

In the current study, we reject the hypothesis that the clinical outcome is worse in a group of patients defined as tissue HER2-positive by Centaur only and not treated with adjuvant HER2-targeted therapy compared to patients defined as HER2-positive by IHC/FISH and treated with adjuvant HER2-targeted therapy. In fact, the best outcome was observed in the group of patients defined as HER2-positive by Centaur only. In contrast to this finding, Konecny *et al* (18) demonstrated an association between HER2 overexpression by ELISA and shorter disease-free survival (DFS) in a cohort of 587 patients with primary breast cancer prior to the era of HER2-targeted therapy. Currently, adjuvant HER2-targeted therapy is the standard of care for HER2-positive breast cancer patients. Scrutiny of the clinically relevant question as to whether we could increase the number of patients eligible for adjuvant HER2-targeted therapy could only be done in a design such as the present one followed by a prospective intervention study.

A number of studies have indicated the existence of an additional group that may benefit from adjuvant HER2-targeted therapy. First, as shown in a previous study [HERceptin Adjuvant (HERA) trial], among patients defined as HER2-positive by IHC and FISH ($IHC2^+$ and $FISH^+$), a significant improvement in clinical outcome was observed in patients treated with adjuvant chemotherapy plus trastuzumab for one year (19). Second, Gilcrease *et al* (20) demonstrated that even a low-level HER2 expression ($IHC1^+$) can be associated with a worse outcome in node-positive patients. Finally, the study by Viale raised an ongoing discussion regarding the misclassification of some patients by FISH using HER2/CEP17 ratio instead of relating the HER2 copy number to the cell count (21).

The cut-off value of 72 ng/mg used in this study is consistent with the cut-off value of 400 fmol/mg (~74 ng/mg) found by Konecny *et al* (18), who optimized the cut-off value to provide the maximum separation of patients according to DFS. On the other hand, Müller *et al* (22) applied ROC statistics to optimize their cut-off value (42 ng/mg) according to the FISH results. If we had used this lower cut-off value, we would have defined 51% of the patients as HER2-positive (193 out of 379 patients). In contrast to these two studies, we aimed to find a novel and more sensitive assay and therefore chose to investigate Centaur HER2 as a biological variable and defined the cut-off value in our study according to the autologous reference tissue by applying a 97.5% CI.

Another unexpected, although interesting finding of this study was that the HER2-positive patients in group 2 (age ≥ 60) had a worse outcome than expected, possibly due to the lack of adjuvant HER2-targeted therapy. This emphasizes the need for a change in clinical practice where many elderly patients

are not selected for adjuvant chemotherapy and HER2-targeted therapy based on age only. Furthermore, Palmieri *et al* (23) reported a worse outcome in patients who were not offered HER2-targeted therapy due to a clinical judgment that the breast cancer was low-risk. Likewise, Tovey *et al* (24) emphasized the need for HER2-targeted therapy even in low-grade, node-negative tumors. Sawaki *et al* (25) showed that elderly patients tolerated trastuzumab well, which highlights the need for a new view of elderly patients and adjuvant HER2-targeted therapy.

In conclusion, the main finding of the present study is that quantitative the detection of HER2 concentration using Centaur does not define a new group of patients eligible for HER2-targeted therapy. Therefore, tissue HER2 status defined by IHC/FISH analysis remains the gold standard. HER2 amplification is presumably the decisive factor, which, in addition to an overexpression of the HER2 protein, leads to the aggressive nature of HER2-positive tumors. Further studies are therefore warranted in order to identify novel methods of detecting this amplification.

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Conflicts of interest

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